Department for Environment, Food and Rural Affairs

Application for consent to release a GMO – organisms other than higher plants

Part A1: Information required under schedule 2 of the Genetically Modified organisms (Deliberate Release) Regulations 2002

Part I: General information

1. The name and address of the applicant and the name, qualifications and experience of the scientist and of every other person who will be responsible for planning and carrying out the release of the organisms and for the supervision, monitoring and safety of the release

ILiAD Biotechnologies, LLC

4581 Weston Road, Suite 260, Weston, FL USA 33331

Chief Medical Officer

PPD medical monitor Telephone (24 hour): +1 888 483 7729

2. The title of the project

Study number IB-202P:

A Phase 2b, Placebo-Controlled, Randomised Study of BPZE1 Intranasal Pertussis Vaccine in Healthy Adults to Assess Protection Against Colonisation Following Challenge with Virulent Wild-Type *Bordetella pertussis*

Part II: Information relating to the organisms

Characteristics of the donor, parental and recipient organisms

3. Scientific name and taxonomy

Genetically attenuated strain of *Bordetella pertussis* (BPZE1)

Name		Details
1.	order and/or higher taxon (for animals):	Bacteria
2.	genus	*Bordetella*
3.	species	*B. pertussis*
4.	subspecies	N/A
5.	strain	Tohama I
6.	pathovar (such as, biotype, ecotype, race)	N/A
Common name		N/A

Abbreviations: N/A, not applicable

4. Usual strain, cultivar or other name

Tohama I *B. pertussis* base strain is genetically modified to create BPZE1.

5. Phenotypic and genetic markers

BPZE1 is based on the *B. pertussis* Tohama I strain, whose genome sequence can be found in Parkhill et al. Nat. Genet 2003;35:32-40. The mother strain of BPZE1 is the streptomycin- and nalidixic acid-resistant Tohama I derivative BPSM, described in Antoine and Locht. Infect. Immun. 1990; 58:1518-1526.

In addition, in the BPZE1 genome the *B. pertussis ampG* gene was replaced by the *Escherichia coli ampG* gene, the pertussis toxin gene was mutated so that the Arg-9 residue and the Glu-129 residues of the S1 subunit were replaced by lysine and glycine, respectively, and dermonecrotic toxin gene was deleted, as described in Mielcarek et al. PLoS Pathog 2006;2: e65. The entire genome sequence of BPZE1 is available on request.

6. The degree of relatedness between the donor and recipient or between parental organisms

The differences between *B. pertussis* Tohama 1 and BPZE1 are:

1. The genomic mutations (chromosomal) that have led to resistance to streptomycin and nalidixic acid in the parent strain.
2. The replacement of the *B. pertussis ampG* gene by that of *E. coli*.
3. The two codon changes in the pertussis toxin S1 subunit gene that have led to genetically detoxified pertussis toxin (*ptx*).
4. The deletion of the dermonecrotic toxin gene (*dnt*).

7. The description of identification and detection techniques

Following the intranasal inoculation of BPZE1 in Phase 1 and 2 clinical studies, nasopharyngeal aspirations or washings were collected as per the laboratory manual and cultured using standard methodologies (Bordet Gengou, Charcoal agar).

Colony growth was assessed by visual inspection for *B. pertussis* followed by confirmation using Matrix-Assisted Laser Desorption or Ionisation-Time of Flight (MALDI-TOF) mass spectrometry (MS) MALDI-TOF and/or polymerase chain reaction (PCR) amplification.

To identify BPZE1 and to distinguish it from wild-type *B. pertussis*, specific PCR amplification can be used that target the *E. coli ampG* gene and the deletion of the dermonecrotic gene, as described in Feunou et al.

Vaccine 2008; 26:5722-5727, as well as quantitative PCR to target the mutations introduced in the pertussis toxin S1 subunit gene as described in Thalen et al. Vaccines 2020;8:523. Phase 2b utilised PCR amplification of upstream and downstream regions of the *dnt* gene and the specific *E. Coli* ampG genes and a similar approach will be utilised in this study (nasopharyngeal swabs or nasal washes)**:**

- *dnt* Forward primer: 5’-TAT AGA ATT CGC TCG GTT CGC TGG TCA AGG-3’
- *dnt* Reverse primer: 5'-TAT AAA GCT TCT CAT GCA CGC CGG CTT CTC-3'
- *E. coli ampG* Forward primer: 5’-ATG TGC TTC CGG CAG AAG AA-3’
- *E. coli ampG* Reverse primer: 5'-CAA GCG TTT TGT TAA CCA CG-3'

8. The sensitivity, reliability (in quantitative terms) and specificity of detection and identification techniques

The specificity of the PCR and quantitative PCR techniques described above is 100%. The PCR sensitivity allows the detection of 10 genome equivalents of BPZE1, and the quantitative PCR targeting the pertussis toxin S1 subunit gene can detect less than 1 wild-type *B. pertussis* per 10^6 CFU of BPZE1 (as described in Thalen et al. Vaccines 2020;8:523).

9. The description of the geographic distribution and of the natural habitat of the organisms including information on natural predators, prey, parasites and competitors, symbionts and hosts

Wild type *B. pertussis* is indigenous to European countries with strain dominance changing over geographies and time due to selection pressures. The Tohama I strain (parental strain of BPZE1) was isolated from a patient in Japan.

It has been sub-cultured *in vitro* since the 1950's and is not found as a circulating strain but is available for laboratory use (https://www.uniprot.org/taxonomy/257313).

Wild-type *B. pertussis* is endemic throughout the world and current disease is controlled with vaccination policies, using acellular pertussis vaccination in the UK. *B. pertussis* is a strictly human pathogen (non-invasive) that colonises the upper respiratory tract of humans and cannot exist outside the host. There are no known intermediaries.

However, mice and other primates can be models for *B. pertussis* investigations as they can be infected through specific inoculation (pre-clinical studies). The *B. pertussis* organism is frequently grown in laboratories within the UK as part of surveillance for epidemic outbreak monitoring.

10. The organisms with which transfer of genetic material is known to occur under natural conditions

The genome of *B. pertussis* (and therefore BPZE1 as well) contains no genetic information of horizontal gene transfer systems and no plasmids. Therefore, horizontal gene transfer to other organisms is virtually impossible, neither in natural conditions, nor in laboratory conditions.

The resistance to streptomycin and nalidixic acid is due to mutations in the bacterial chromosome, which cannot be transferred to other organisms.

11. Verification of the genetic stability of the organisms and factors affecting that stability

Genetic stability of BPZE1 after 20 and 27 weeks of continuous passaging *in vitro* and *in vivo*, respectively, has been demonstrated.

When compared to non-passaged BPZE1, three modified loci, *ampG*, *dnt* and *ptx*, remained unchanged after the different passages. The *B. pertussis ampG* and *dnt* genes

remained absent, the *E. coli ampG* gene was invariably present, and the two mutations in the *ptx* gene resulting in the R9K and E129G substitution of the pertussis toxin S1 subunit were stable.

Furthermore, the microarray analysis indicated that all the genes present before passaging were still present after 20 *in vitro* or 9 *in vivo* passages. Similarly, after 20 *in vitro* or 9 *in vivo* passages, the protective activity of BPZE1 remained comparable to that of non-passaged BPZE1 (Feunou-Feunou, P et al. 2008. Vaccine 26:5722-5727).

The global genomic stability of BPZE1 did not appear to be affected over the period examined in the above referred study, which should be sufficient to consider that genetic stability of this vaccine strain is not a serious concern for further development of BPZE1 as a live attenuated vaccine candidate against whooping cough.

12. The following pathological, ecological and physiological traits:

a. the classification of hazard according to existing Community rules concerning the protection of human health and the environment

The parent *B. pertussis* organism is the risk group 2 pathogen (HSE List of biological Agents) due to its ability to induce disease in humans. As BPZE1 is not disease inducing and the BPZE1 risk group has been downgraded and activities permitted for biosafety level 1 in France, Netherlands, Belgium, Spain, Germany, U.S., and Sweden.

b. the generation time in natural ecosystems, and the sexual and asexual reproductive cycle

The organism is asexual, therefore, reproduction occurs through cell division, which corresponds to a generation time of approximately 4 hours in optimal laboratory conditions.

The Bordetella species have fastidious growth requirements and cannot survive outside the human host for significant periods. In laboratory settings with *B. pertussis* microbiologic growth evidence on inanimate surfaces is less than 6 days. There is no documentation of *B. pertussis* on animate objects in community settings and it does not exist on plants, in water, or in soil.

c. information on survivability, including seasonability and the ability to form survival structures, including seeds, spores and sclerotia

B. pertussis does not exist in the environment. In laboratory settings and under stringent growth conditions (*B. pertussis* is a fastidious organism), the relevant factors affecting survivability, include nutrients in the culture medium and temperature. The organism survives optimally at approximately 37ºC.

The survival time in phosphate buffer at room temperature is 18 hours. *B. pertussis* does not produce seeds, spores or sclerotia. In the natural environment, virulent *B. pertussis* infections can occur throughout the year through close contacts (human to human transmission), epidemic surges are slightly more common in the spring and summer months but can be found across seasons and geographies and is more likely associated with the loss of immunity.

Currently acellular vaccines do not protect against acquiring *B. pertussis* infection through human to human contact and the rise in *B. pertussis* epidemics is thought to be at least partially due to the sole use of acellular vaccines.

d. pathogenicity, including infectivity, toxigenicity, virulence, allergenicity, carrier (vector) of pathogen, possible vectors, host range including non-target organisms and possible activation of latent viruses (proviruses) and ability to colonise other organisms

B. pertussis is a strictly human pathogen and does not spread to other live organisms (no intermediary hosts). Other animals, including laboratory mice and baboons (best models available), can be infected with high doses of wild-type organism as demonstrated in challenge studies.

They clear the infection spontaneously. BPZE1 can likewise be acquired by nasal inoculation but without evidence of disease manifestations due to the removal or modification of the three key toxin-mediated genes (pertussis toxin, tracheal cytotoxin and dermonecrotic toxin).

The modifications in BPZE1 are specifically designed to remove the virulence of the parental strain and therefore do not lead to disease manifestations. BPZE1 is a live attenuated vaccine and able to induce mucosal and systemic immunity through natural mucosal pathways.

Its potential is unique from acellular pertussis vaccines which do not work on infection or acquisition, but rather only prevent lower airway disease manifestations.

By targeting protection against acquisition (infection) of wild-type *B. pertussis,* BPZE1 should contribute significantly to abrogate human to human transmission of wild-type *B. pertussis* while also averting lower airway disease manifestations.

Clinical studies, including so far 356 human subjects exposed to BPZE1, have shown that high doses of BPZE1 (up to 10^9 CFU) do not induce whooping cough or related disease in humans. The GMO is strongly attenuated and does not induce airway inflammation and, in fact, protects against airway inflammation induced by allergens or viral infections, as documented in pre-clinical studies (for a summary see Cauchi and Locht. Front. Immunol. 2018;9:2872).

e. antibiotic resistance, and potential use of these antibiotics in humans and domestic organisms for prophylaxis and therapy

BPZE1 is a derivative of the streptomycin- and nalidixic acid-resistant Tohama I strain (this strain was later named BPSM and is the direct precursor of BPZE1). Resistance is due to mutations in the chromosome, and not to a plasmid or a phage, and therefore cannot be transmitted to other micro-organisms due to the lack of plasmids and genetic information necessary for horizontal gene transfer. BPZE1 has been fully sequenced, and the location of the mutations is known.

Clinically *B. pertussis* infection is treated with macrolides as first line therapy. Neither streptomycin or nalidixic acid are used against clinical *B. pertussis*. Effective antibiotic treatment with azithromycin could be given in case of accidental transmission to other humans. Alternative treatment with trimethoprim sulfamethoxazole can be given in the case of macrolide allergy.

f. involvement in environmental processes including primary production, nutrient turnover, decomposition of organic matter and respiration

BPZE1 is grown in laboratory-controlled conditions using fully synthetic medium. There is no deliberate release in the environment of the BPZE1 organism, nor of the culture medium after BPZE1 growth.

13. The sequence, frequency of mobilisation and specificity of indigenous vectors and the presence in those vectors of genes which confer resistance to environmental stresses

BPZE1 does not contain any phage, plasmid or transposable vector.

Antibiotic resistance genes are integrated into the chromosome and are not expressed in genetically mobile elements or inserted into prophages. As discussed above, genetic exchange of chromosomal genes with other *B. pertussis* is possible, but highly improbable. Release of genes which confer resistance to environmental stresses is therefore excluded de facto.

14. The history of previous genetic modifications

BPZE1 is a derivative of *B. pertussis* BPSM, which is itself a derivative of *B. pertussis* Tohama I, a natural clinical isolate from a Japanese patient with whooping cough. Since the 1950's Tohama 1 strain has been sub-cultured in laboratory environments and has not been in circulation.

BPSM was selected for its resistance to streptomycin and nalidixic acid as described in Antoine and Locht. Infect. Immun. 1990; 58:1518 to 1526. No other genetic changes were introduced in BPSM.

Characteristics of the vector

15. The nature and source of the vector

A plasmid vector was used for transformation, but no plasmid material is left in the final strain.

16. The sequence of transposons, vectors and other non-coding genetic segments used to construct the genetically modified organisms and to make the introduced vector and insert function in those organisms

No transposon was used to construct BPZE1. To construct BPZE1, the *B. pertussis ampG* gene was first replaced by *E. coli ampG* using allelic exchange. A PCR fragment of the *B. pertussis* genome(http://www.sanger.ac.uk/Projects/B_pertussis/), upstream of the *B. pertussis ampG* gene, was amplified using *B. pertussis* BPSM genomic DNA as template. This fragment was inserted into a suicide vector containing the *E. coli ampG* gene with flanking *B. pertussis* DNA.

The resulting plasmid was conjugated with BPSM, and two successive homologous recombination events were selected as described in Stibitz. Methods Enzymol. 1994; 235:458-465. The *B. pertussis* strain containing *E. coli ampG* and lacking *B. pertussis ampG* was then selected, and the entire *ampG* locus was sequenced. This strain was then used for further engineering.

The *ptx* genes were deleted from the chromosome of this strain as described in Antoine & Locht. Infect. Immun. 1990; 58:1518-1526, and then replaced by mutated *ptx* coding inactive PTX. The *Eco*RI fragment containing the mutated *ptx* locus from pPT-RE (Alonso et al. Infect. Immun. 2001; 69:6038-6043) was inserted into the *Eco*RI site of pJQ200mp18rpsl (Antoine et al. J. Mol. Biol. 2005; 351:799-809.

The resulting plasmid was integrated into the *B. pertussis* chromosome at the *ptx* locus by homologous recombination after conjugation via *E. coli* SM10. The *ptx* locus in the chromosome of the resulting *B. pertussis* strain was sequenced to confirm the presence of the desired mutations.

Finally, the *dnt* gene was deleted from the resulting *B. pertussis* strain. The *dnt* flanking regions were amplified by PCR using BPSM genomic DNA as template and oligonucleotides for the *dnt* upstream region and for the *dnt* downstream region as primers. The resulting DNA fragments were linked together using the Fast Link kit (Epicentre Biotechnologies, Madison, WI).

The ligated fragment was then inserted into pCR2.1-Topo (Invitrogen) and inserted into the unique *Eco*RI site of pJQmp200rpsL18. The resulting plasmid was introduced into *B. pertussis* by conjugation via *E. coli* SM10. The *dnt* locus of this final strain, named BPZE1 was sequenced.

No vector DNA was present in the final BPZE1 construction.

17. The frequency of mobilisation, genetic transfer capabilities and/or methods of determination of the inserted vector

As described above (item #16), no vector DNA was present in the chromosome of BPZE1.

18. The degree to which the vector is limited to the DNA required to perform the intended function

The vector for the addition of the DNA required to perform the intended function was a ‘suicide vector’, used only for the construction of BPZE1 via two steps of homologous recombination and, as the result of the second homologous recombination leading to the construction of BPZE1, the vector DNA was completely eliminated in the final BPZE1 strain.

Characteristics of the modified organisms

19. The methods used for the modification

The genetic modifications alter or remove three *B. pertussis* toxins, PTX, TCT and DNT. This strain, named BPZE1, consequently expresses an enzymatically inactive PTX by altering two key amino acids for the enzymatic activity of the toxin (mutations R9K and E129G; either one of these mutations abolishes toxin activity), shows a 100-fold reduction in TCT activity by the replacement of the *B. pertussis ampG* gene by that of *E. coli* and does not produce DNT by the deletion of its structural gene.

The genetic modifications in BPZE1 strongly increase the *in vivo* and *in vitro* safety:

- The double nucleotide mutation in the substrate binding and the active site of the PT results in a strong reduction of the enzyme activity.
- The replacement of the *B. pertussis ampG* gene by the *Escherichia coli ampG* gene results in an over 99% reduction in release of the TCT in the medium.
- The DNT is not expressed in the BPZE1 strain.
- BPZE1 is not invasive and has no selective advantage in the environment. The potential for exchange of genetic material is virtually nonexistent since *B. pertussis* does not harbor plasmids or conjugative transposons. In addition, *B. pertussis* Tohama I (origin of BPZE1) does not harbor intact prophage genomes and is therefore incapable of producing functional phage particles.

The detailed method used for the modification is described in item #16.

20. The methods used

a. to construct inserts and introduce them into the recipient organism

The insert encodes a functional AmpG transporter protein. *B. pertussis* AmpG is inefficient in the internalisation of peptidoglycan breakdown products, such as the TCT. The *B. pertussis ampG* gene was therefore replaced by *E. coli ampG*. The resulting strain expressed less than 1% residual TCT activity (background activity).

The detailed method used for this construct is described in item #16.

b. To delete a sequence

The *dnt* gene is deleted from the chromosome. Please refer to item #16 for further details

21. The description of any insert and/or vector construction

The deleted genes are all required for full virulence. The final construct contains no vector DNA. The only foreign DNA insert in BPZE1 is the *E. coli ampG* gene, and the precise method used for this insertion is described in item #16.

22. The purity of the insert from any unknown sequence and information on the degree to which the inserted sequence is limited to the DNA required to perform the intended function

The insert of foreign DNA only contains the sequence of the *E. coli ampG* gene, with no unknown sequence.

23. The methods and criteria used for selection

The methods used for selection was based on the antibiotic resistance phenotype of the selected strains. BPSM, the mother strain of BPZE1, is resistant to streptomycin and nalidixic acid due to mutations in the chromosome of BPSM.

To construct BPZE1 via conjugation with transformed *E. coli* SM10, BPSM and the various intermediate strains were cultured on Bordet-Gengou blood agar plates containing nalidixic acid to counter-select against *E. coli* and gentamycin to select for integration of the non-replicative plasmid into the *B. pertussis* chromosome by homologous recombination.

The resulting NalR GenR strains were then purified and plated onto Bordet-Gengou blood agar plates containing streptomycin to select for the second event of homologous recombination, which eliminates the vector DNA and only maintains the desired insert. The final strain does not contain the GenR gene anymore and is therefore sensitive to gentamycin.

24. The sequence, functional identity and location of the altered, inserted or deleted nucleic acid segments in question and, in particular, any known harmful sequence

There are no known harmful sequences in BPZE1. The *E. coli ampG* gene was inserted at the *ampG* locus of *B. pertussis* by virtue of allelic exchange using the *B. pertussis ampG* gene flanking regions for homologous recombination.

Characteristics of the genetically modified organisms in their final form

25. The description of genetic traits or phenotypic characteristics and in particular any new traits and characteristics which may be expressed or no longer expressed

BPZE1 produces less than 1% of the wild-type level of TCT, does not produce DNT and produces a genetically inactivated PT. The two codons altered in the pertussis toxin S1 gene result in the production of a PT analogue which no longer expresses enzymatic ADP-ribosyl transferase activity, thereby abolishing its toxic activity which still allows for the needed immunologic response against the natural toxin.

The GMO grows slightly less well in the respiratory tract of adult mice than the parent strain BPSM.

26. The structure and amount of any vector or donor nucleic acid remaining in the final construction of the modified organisms

There is no vector DNA remaining in the BPZE1 chromosome.

27. The stability of the organism in terms of genetic traits

Genetic stability of BPZE1 after 20 and 27 weeks of continuous passaging *in vitro* and *in vivo*, respectively, has been demonstrated (See Feunou-Feunou, P et al. 2008. Vaccine 26:5722-5727).

Twenty *in vitro* continuous passages over a period of 20 weeks and 9 *in vivo* passages through mice over a period of 27 weeks have shown that BPZE1 is genetically stable both *in vitro* and *in vivo* and maintains its protective properties after passages (Feunou et al 2008 Vaccine 26:5722-5727).

28. The rate and level of expression of the new genetic material in the organisms and the method and sensitivity of measurement of that rate and level

Since TCT production by the GMO is below background level (such as, less than 1% of wild-type levels) and since TCT may be involved in the cough syndrome, the GMO is expected to disseminate much less efficiently than the recipient strain. Severe or prolonged cough has not been reported in phase 1 or 2 clinical trials.

29. The activity of the gene product

No DNT is produced by BPZE1 and TCT levels are less than 1% of wild-type levels. PT is enzymatically inactive and therefore no longer acts as a toxin, yet it is produced as an immunogen and induces anti-PT antibodies in vaccinated subjects as described in Thorstensson et al. PLoS One 2014;9: e83449, Jahnmatz et al. Lancet Infect Dis 2020;20:1290 to 1301 and Lin et al. J. Clin. Invest. 2020 ;130 :2332 to 2346.

30. The description of identification and detection techniques, including techniques for the identification and detection of the inserted sequence and vector

The GMO is identified by genetic characterisation using PCR and sequencing techniques. PCR analysis of the *ampG* and *dnt* loci of BPZE1: Genomic DNA is extracted from isolated colonies and used as template for the PCR using appropriate sense and anti-sense oligonucleotides (see Feunou et al. Vaccine 2008; 26:5722-5727).

The amplified products were analysed by electrophoresis within a 1% agarose gel in Tris-acetate-EDTA (TAE) buffer containing ethidium bromide and visualised under UV light.

Sequence analysis of the *ptx* locus of BPZE1: The DNA fragments containing the region encompassing the R9K and the E129G mutations of the *ptx*S1 gene are amplified by quantitative PCR from bacterial genomic DNA, using appropriate primers as described in Thalen et al. Vaccines 2020;8:523.

31. The sensitivity, reliability (in quantitative terms) and specificity of detection and identification techniques

The specificity of the PCR and quantitative PCR techniques described above is 100%. The PCR sensitivity allows the detection of 10 genome equivalents of BPZE1, and the quantitative PCR targeting the pertussis toxin S1 subunit gene is able to detect less than 1 wild-type *B. pertussis* per 10^6 CFU of BPZE1 (as described in Thalen et al. Vaccines 2020;8:523).

32. The history of previous releases or uses of the organisms

BPZE1 has undergone five human trials as a pertussis vaccine candidate in the United States, Sweden and the United Kingdom.

- Phase 1a study BT0604, NCT01188512, EudraCT # 2010-019936-11
- Phase 1b study C14-80, NCT02453048, EudraCT # 2015-001287-20
- Phase 2a study 17-0010, NCT03541499
- Phase 2b study IB-200P, NCT03942406
- Phase 2b study IB-201P, NCT05116241, EudraCT #2020-005937-34

The results of two of them have been published: Thorstensson et al. PLoS One 2014;9:e83449 and Jahnmatz et al. Lancet Infect Dis 2020;20:1290-1301.

The phase 2b clinical study IB-200P report has been submitted to the FDA by ILiAD Biotechnologies (IND :18140) and the phase 2a report will be submitted later this year by the Division of Microbiology and Infectious Diseases (NIH) (IND: 18215). The Phase 2b study, IB-201P, is currently ongoing.

Further, there have been multiple non-clinical studies conducted with the GMO as detailed below:

Study number	**BPZE1 dose (CFU)**	**Design**	**Organ evaluated**
Study number 1 Lung Colonisation Kinetics	10^6 10^5 10^4 10^3 10^2	Groups of 20 3-week-old (infant) and 8-week-old (adult) female Balb/C mice were intranasally immunised with a range of BPZE1 doses or wild-Type *B. pertussis*. Groups of 10, 3-week-old (infant) and 8-week-old (adult) female Balb/C mice were vaccinated and challenged with wild-type B. pertussis 2-months after vaccination, comparing wild-type *B. pertussis* colonisation in BPZE1 vaccinated versus unvaccinated mice.	Lungs, Blood
Study number 2 Protective immunity against *B. pertussis* and B. parapertussis	10^6	Groups of 8 3-week-old (infant) and 8 8-week-old (adult) female Balb/C mice were intranasally immunised with BPZE1 or aPV and challenged with wild-type *B. pertussis* or B. parapertussis 2-mo. post-vaccination	Lungs, Blood
Study number 3 Immunodepressed mice	10^{10}	Groups of 18 2 to 7 day-old (neonatal) and 6 to 8-week-old (adult) IFN-γR KO or 129/Sv mice were immunised through a nebuliser with BPZE1 or wild-type *B. pertussis*	Lungs, Liver
Study number 4 Allergic Airway Inflammation Model	10^6	Groups of more than 10 8 to12 week-old (adult) Balb/C mice were immunised via nebuliser with BPZE1 or wild-type *B. pertussis*. Mice were sensitised to ovalbumin (OVA) in adjuvant at days 24, 35, 36, and 37.	Lungs, Blood

Study number	**BPZE1 dose (CFU)**	**Design**	**Organ evaluated**
Study number 5 Genetic stability	10^6	Groups of 6 3 week-old (infant) and 8-week-old (adult) female Balb/C mice were intranasally immunised with BPZE1. 2-weeks post-vaccination mice were sacrificed, and lung homogenate used to infect another group of mice, with the procedure repeated 9 times.	Lungs
Study number 6 Toxicity of *B. pertussis* BPZE1 vaccine after repeated nasal administration in young mice – a follow-up study	10^6	8 groups of 10 4-week-old (young) NMRI mice were intranasally immunised with 10^6 CFU BPZE1 3 times at 2-week intervals and analysed at 21 days after the last vaccination.	Lungs
Study number 7 Pyrogenicity and Ocular Toxicity in Rabbit	10^7 10^6	2 groups of 5 female NZW rabbits were immunised intranasally with 10^7 CFU BPZE1 or intra-ocularly with 10^6 CFU BPZE1 and analysed at day-4.	Eye
Study number 8 Non-human primate study	10^9 10^{10}	3 groups of 3 to 4 juvenile baboons were vaccinated with 1 millilitre (ml) intra-nasal and 1ml intra-tracheal of 10^9 CFU BPZE1 or 10^{10} CFU BPZE1 or not vaccinated (naïve), and were challenged with 1ml intra-nasal and 1ml intra-tracheal of 7.5 by 10^9 CFU of wild-type D420 *B. pertussis* 7-weeks after vaccination	Nasal Aspirate, Blood

Study number	BPZE1 dose (CFU)	Design	Organ evaluated
Study number 9 Non-human primate pilot study	10^9	Phase1, 3 healthy neonate baboons, vaccinated between 6 to 8 days after birth with BPZE1 lyophilised drug product via VaxINator. Mothers also assessed for BPZE1 carriage following vaccination (for example, transmission)	Nasal Aspirate, Blood

33. In relation to human health, animal health and plant health

a. the toxic or allergenic effects of the non-viable organisms and/or their metabolic products

No toxic or allergic effect of BPZE1 has been detected. BPZE1 has anti-allergy properties as described in Kavanagh et al. Clin. Exp. Allergy 2010; 40:933-941, Li et al. Allergy 2012;67:1250-1258.

b. the comparison of the organisms to the donor, recipient or (where appropriate) parental organism regarding pathogenicity

In contrast to the parental organism, the GMO is strongly attenuated and does not induce airway inflammation and conversely protects against airway inflammation induced by allergens or viral infections, as described in Kavanagh et al. Clin. Exp. Allergy 2010; 40:933-941, Li et al. Allergy 2012;67:1250-1258, Li et al. J. Virol. 2020; 84:7105-7113 and Cauchi and Locht Front. Immunol. 2018; 9:2872).

Furthermore, in contrast to the parental organism, the GMO does not induce whooping cough or related disease in non-human primates, as shown in Locht et al. J. Infect. Dis. 2017;216:117-124, nor in humans, as shown in 4 clinical trials (see Thorstensson et al. PLoS One 2014;9:e83449 and Jahnmatz et al. Lancet Infect Dis 2020;20:1290-1301).

c. the capacity of the organisms for colonisation

The GMO grows slightly less well in the respiratory tract of adult mice. It is able to transiently colonise the respiratory tract of baboons, as shown in Locht et al. J. Infect. Dis. 2017;216:117-124, and of humans, as shown in Thorstensson et al. PLoS One 2014;9:e83449 and Jahnmatz et al. Lancet Infect Dis 2020;20:1290-1301.

Colonisation in the human respiratory tract with the highest dose tested so far (10^9 CFU) lasts generally for 2 to 3 weeks.

d. if the organisms are pathogenic to humans who are immunocompetent

Studies in mice demonstrated that BPZE1 is non-pathogenic in severely immunocompromised mice, such as IFN-gR KO mice, as shown in Skerry et al. Clin. Vaccine Immunol. 2009; 16:1344-1351, and in MyD88 KO mice, as shown in Debrie et al. J. Immunol. 2019; 203:3293-3300.

i. diseases caused and mechanisms of pathogenicity including invasiveness and virulence

The GMO has been demonstrated to be safe in 356 subjects who have received product over 4 clinical trials with no vaccine-related serious adverse events and similar reactogenicity as placebo controls. Furthermore, wild-type *B. pertussis* is a strictly mucosal pathogen (non-invasive) and does not disseminate beyond the respiratory mucosa.

In very rare occasions other Bordetella species have been noted to cause disseminated disease in severely immunocompromised humans (for example, *Bordetella bronchiseptica* and *Bordetella hinzii*) (reference Cookson JCM 1994).

In mouse models high doses of *B. pertussis* can disseminate in liver and spleen in IFN-gR KO mice. However, even in this model BPZE1 does not disseminate outside of the respiratory tract, as shown in Skerry et al. Clin. Vaccine Immunol. 2009; 16:1344-1351.

ii. communicability

B. pertussis is transmitted in humans through natural infection or acquisition and in non-human primate models following challenge. *B. pertussis* is not transmittable in mouse models.

In the baboon model, *B. pertussis* has been shown to be transmittable from one baboon to a co-housed adjacent baboon, even in the face of recent acellular pertussis vaccination as shown in Warfel et al. Proc. Natl. Acad. Sci. USA 2014;111:787-792. In contrast to acellular vaccination, BPZE1 vaccination protected baboons from developing whooping cough and harboring the wild type of bacterium in the respiratory tract (Locht et al JID 2017).

ILiAD Biotechnologies is developing a neonate baboon model and currently a pilot is ongoing: 7-days old neonate baboons (n=3) were vaccinated with BPZE1 and then co-housed with their mothers.

As expected, BPZE1 organisms could be detected in the nasopharynx of neonatal baboons with subsequent immunity induction. However, close contact with mothers did not demonstrate BPZE1 detection in the maternal nasopharynx during the time of detection in the infants. Considering the very close contacts between the mothers and their offspring, these observations indicate that communicability of BPZE1 is at best very low.

iii. infective dose

The Phase 1b study demonstrated 80% colonisation rates at 10^7 to 10^9 CFU dose with 400µl per nostril administration.

The Phase 2b study showed a single vaccination (10^9 CFU) protected against attenuated challenge with BPZE1 3 months later (less than 10% colonised) but vaccination with acellular Boostrix was unable to protect against acquisition 3 months later (70% acquisition).

Clearance of BPZE1 has been demonstrated in naïve and recently acellular vaccinated individuals in the normal course of 2 to 3 weeks.

iv. host range and possibility of alteration

B. pertussis is a strictly human pathogen. The genetic modifications (replacement of the *ampG* gene, deletion of the DNT gene, and the mutations of the PT gene) are not expected to alter the host range of *B. pertussis* BPZE1 compared to the wild-type *B. pertussis*.

v. possibility of survival outside of human host

Unlike *B. bronchiseptica*, *B. pertussis* bacteria can only survive outside of a human host for a limited duration. Compared to the relative survival of *Bacillus subtilis* in aerosols, *B. pertussis* survival is reduced by more than 90% and loses more than 95% of its survival in aerosols within 30 min.

Only in intense microbiology laboratory conditions can *B. pertussis* be retrieved off inanimate objects during limited periods of time. Such retrieval has not been demonstrated in non-laboratory settings.

vi. presence of vectors or means of dissemination

There is no vector DNA in BPZE1 and therefore no vector dissemination.

vii. biological stability

Genetic stability of BPZE1 after 20 and 27 weeks of continuous passaging *in vitro* and *in vivo*, respectively, has been demonstrated (See Feunou-Feunou, P et al. 2008. Vaccine 26:5722-5727)

viii. antibiotic-resistance patterns

BPZE1 is resistant to streptomycin and to nalidixic acid, but sensitive to macrolides routinely used in the control of *B. pertussis* infections.

ix. allergenicity

The GMO is strongly attenuated, does not induce airway inflammation and, in fact, protects against airway inflammation induced by allergens or viral infections.

X. availability of appropriate therapies

BPZE1 is an attenuated live organism and is not pathogenic. In case of accidental exposure, an efficient treatment against *B. pertussis* is commercially available and is based on administering azithromycin or another macrolide. Alternative treatment with trimethoprim sulfamethoxazole can be given in the case of macrolide allergy.

e. Other product hazards

N/A

Part III: Information relating to the conditions of release

The release

34. The description of the proposed deliberate release, including the initial purpose or purposes of the release and any intention to use the genetically modified organisms as or in a product in the future

The GMO, BPZE1, is a live attenuated *B. pertussis* intranasal vaccine that is being studied in a clinical trial in healthy adults as a follow-up to a previously successful Phase 2 study in 300 adults.

This Phase 2b virulent challenge study will investigate colonisation rates, immunologic response and the safety of BPZE1 vaccination to potentially protect against colonising wild-type *B. pertussis* infection in healthy adults using a virulent wild-type challenge model.

By stopping initial infection of wild-type *B.pertussis* through mucosal mediated pathways of immunity, long term consequences of toxin mediated disease and transmission to others in the community can be avoided. The commercial opportunity is to gain regulatory licensure to vaccinate adults and children against pertussis, and perhaps eventually immunise infants.

35. The intended dates of the release and time planning of the experiment including frequency and duration of releases

The clinical study has a planned initiation of April 27, 2022. The subjects in the study will receive a single dose of either BPZE1 or placebo. Nasal administration into both nostrils of each study volunteer will be achieved via a mucosal atomiser (spraying) device, with delivery of 400 μl volume of liquid vaccine into each individual's nostril being accomplished in less than 5 seconds. Therefore, the pharmacy preparation and vaccination timing are expected to be April 27, 2022, through January 2024 at the UK site.

36. The preparation of the site before the release

Preparation of the investigational product will take place in an approved pharmacy like environment at the clinical trial sites. The administration of the investigational product will be prepared by authorised trained personnel at the study sites, according to good clinical practice and the study protocol. Appropriate personal protective equipment will be utilised during preparation and during administration.

Given the ongoing COVID-19 pandemic nasal administration and nasal sampling will also require protective equipment to be worn by clinical staff during these procedures.

Unblinded personnel will prepare the BPZE1 or Placebo. The work area will be clean and only the material required for preparation step will be present. Dose preparation will be performed using appropriate aseptic techniques. Appropriate plan will be in place to administer the dose to the patient within 3 hours after reconstitution. Unblinded staff will not be involved in study-related assessments or have subject contact for data collection following study vaccine administration.

A Material Safety Data Sheet (MSDS) or equivalent document describing occupational hazards and recommended handling precautions either will be provided to the investigator, where this is required by local laws, or is available upon request from sponsor.

We believe that research conducted within this framework adequately mitigates the risks of such research to the public health and therefore no additional measures will be undertaken. Only qualified personnel who are familiar with procedures that minimise undue exposure to themselves and to the environment will undertake the preparation, handling and safe disposal of BPZE1.

37. The size of the site

The GMO is released in a clinical examination room setting. Participants will remain in clinic for 30 minutes of observation following vaccinations before to be discharged to the community returning to the clinic for study visits throughout the trial period.

38. The method or methods to be used for the release

The GMO is reconstituted in the pharmacy or drug area of the clinic from the sealed glass vial and then drawn up into a syringe with a luer-lock tip. The syringe will be capped using a luer-lock capping mechanism for transport to the vaccination room. Just prior to vaccination, the syringe tip is connected to the conical-shape spraying atomiser (mucosal atomiser device (MAD)).

The MAD is designed to fit snuggly around the nostril opening. The subject reclines at 45 degrees or sits with neck hyper-extended.

The tip is inserted into the study volunteer’s nostril and the syringe plunger is briskly compressed to deliver around half the vaccine into the nostril. This is repeated in the second nostril.

The subject remains in their recumbent position for 30 seconds. A fine mist is created by the atomisation device to deliver uniform GMO into the volunteer’s nasopharynx. The use of the MAD allows a good seal and uniform dispersion across the mucosal surface. The chances of significant release to the environment are low due to these procedures.

39. The quantity of organisms to be released

800 μl volume of liquid vaccine (400 μl in each nostril of individual study volunteers).

40. The disturbance of the site, including the type and method of cultivation, mining, irrigation, or other activities

N/A

41. The worker protection measures taken during the release

The primary mode of containment during the administration procedure is application of Standard or Universal Precautions for infectious materials. Personnel handling the GMO during reconstitution will wear disposable gloves, and any other required personal protective equipment based on GMP SOPs in place. The surfaces will be clean, prior to and after performing reconstitution activities in the pharmacy or drug accountability room.

Persons handling the BPZE1 bacteria for vaccination will use personal protective equipment (PPE) and must wash their hands with a suitable disinfecting soap after administration. Similar PPE will be utilised during nasal sampling. Effective antibiotic treatment with azithromycin could be given in case of accidental transmission to other humans, however, BPZE1 is an attenuated live organism and is not pathogenic.

Labs for processing clinical samples, such as, bloods would use standard precautions for bodily fluids.

42. The post-release treatment of the site

Participants:

Nasal (mucosal secretion sample) and blood samples for immunogenicity assessments will be collected before vaccination and at selected time points following study vaccination. BPZE1 has not been extracted from nasal mucosal synthetic absorptive matrix (SAM) samples (data on file, Phase 2b) and BPZE1 is not carried in blood. Normal biohazard precautions will be taken for both of these sample types.

Nasal wash will be collected for *B. pertussis* culture analysis at selected time points following study vaccination to assess initial colonisation and clearance (Day 52 relative to Vaccination Phase) and during the Challenge Phase to assess colonisation with virulent wild-type *B. pertussis* (Days 9, 11 and 14 relative to Challenge Phase).

The safety data on subjects will be collected through the end of the study Day 180 post-vaccination or Day 90 post-challenge, whichever is longer.

Study site

This is a randomised observer blinded study and in accordance with all applicable regulatory requirements, the designated site staff responsible for unblinded drug reconstitution and accountability will maintain investigational product accountability records throughout the course of the study.

Any unused clinical study Investigational Product supplies remaining at the end of the study will be documented and destroyed at site (or returned to depot for destruction) according to local procedures.

Dispensing errors of any kind will be thoroughly documented in the patient's source documentation. Any associated packaging materials will be discarded after use, unless otherwise required due to local requirements.

43. The techniques foreseen for elimination or inactivation of the organisms at the end of the experiment or other purposes of the release

The following techniques or procedures are used for the elimination or inactivation of the GMO at the end of experiment:

1. After reconstitution of the vial, the preparation area is cleaned with 70% alcohol.
2. The used syringe and mucosal atomiser device are discarded into a biohazard container and destruction processes for GMO substances will be followed.
3. Any unused clinical study Investigational Product supplies remaining at the end of the study should be documented and destroyed at site (or returned to depot for destruction) according to local procedures for GMO products.

44. Information on, and the results of, previous releases of the organisms and in particular, releases on a different scale or into different ecosystems

The BPZE1 liquid formulation was initially developed and studied in two Phase 1 clinical trials in Sweden. The product has been further optimised to have longer stability through lyophilisation (freeze drying), has been utilised in two subsequent Phase 2 clinical trials conducted in healthy adults in the United States (US) under US IND, and is currently being used in a Phase 2b clinical trial in healthy school-age children in the United Kingdom and in the US under US IND.

To date, 356 healthy volunteers (18 through 50 years old) have been enrolled and exposed to BPZE1 in completed studies. Reconstitution and delivery in all studies was conducted in a pharmacy or clinic research trial setting with appropriate procedures for clinical trials in place.

None of the study subjects have experienced any vaccine-related serious adverse events. There are no reports of transmission to clinical staff (including those reconstituting or administering the vaccine) or the study subject's close contacts (for example, family members), and no adverse events have been reported by study staff or close contacts (note: information was not proactively sought beyond study participants).

The GMO has also been released in a broad range of animal experiments in mice, rabbits, and primates, as well as *in vitro* growth media or cell culture plates. The environment was scientific laboratories and animal laboratories.

There have been no adverse events identified in the test subjects or scientists conducting experiments. There is no indication of possible undesirable effects on the environment.

The environment (both on the site and in the wider environment)

45. The geographical location and national grid reference of the site or sites onto which the release

The GMO has been released in completed and ongoing human clinical studies in Birmingham (UK), Oxford (UK), Cambridge (UK), Manchester (UK), Liverpool (UK), Bradford (UK), London (UK), Leicester (UK), Bristol (UK), Southampton (UK), Cardiff (UK), Stockholm (Sweden); Nashville, TN (USA), Houston, Texas (USA), Salt Lake City, Utah (USA), and Cleveland, Ohio (USA). The GMO has been released during *in vivo* and *in vitro* experiments in Lille (France), Dublin and Maynooth (Ireland), Singapore, London (UK), Bilthoven (Netherlands), Brussels (Belgium), and Rome (Italy).

The planned clinical trial (Ph-2b study IB-202P) will be conducted at the following site in England:

Site Name	Address
University Hospital Southampton NHS Foundation Trust	Tremona Road Mail point 218, Level C West Wing, NIHR Clinical Research Facility, Southampton, Hampshire, SO16 6YD

46.The physical or biological proximity of the site to humans and other significant biota

Human volunteers and clinicians participating in the release of the GMO at different study sites (refer to item # 45 for site information in England).

47. The proximity to significant biotopes, protected areas or drinking water supplies

N/A

48. The climatic characteristics of the region or regions likely to be affected

N/A

49. The geographical, geological and pedological characteristics

N/A

50. The flora and fauna, including crops, livestock and migratory species

N/A

51. The description of target and non-target ecosystems likely to be affected

The target ecosystem used by *B. pertussis* is the human upper respiratory system. There is no known environmental reservoir for *B. pertussis* (for example, plants, soil or water). The GMO colonises the upper respiratory epithelium without dissemination of the bacteria outside the respiratory tract, which is similar to wild-type *B. pertussis* organism.

This excludes systemic bacteremia of the BPZE1 strain. Wild type *B. pertussis* is spread mainly by aerosol formed by coughing of infected persons or by contact with respiratory secretions directly. Significant coughing is thought to be induced by the TCT, which is more than 99% reduced in BPZE1. The BPZE1 has not been shown to induce significant coughing through active collection of post vaccination reactogenicity through 7 days.

There is no evidence of delayed cough induction beyond the early period of reactogenicity monitoring, therefore, the potential for transmission appears negligible. However, formal transmission studies have not been conducted. The Bordetella species have fastidious growth requirements and have limited survival time outside the human body, so it is highly unlikely that it will affect another ecosystem.

52. The comparison of the natural habitat of the recipient organisms with the proposed site or sites of release

The human respiratory system is the natural habitat of *B. pertussis*. Study participants who consent to the clinical trial will be the recipient of an attenuated BPZE1, a non-pathogenic bacterium.

53. Any known planned developments or changes in land use in the region which could influence the environmental impact of the release

N/A

Part IV: Information relating to the interactions between the organisms and the environment

Characteristics affecting survival, multiplication and dissemination

54. The biological features which affect survival, multiplication and dispersal

Survival and multiplication

The GMO can only survive and multiply in the upper respiratory tract of humans. There is no known external reservoir in the ecosystem for BPZE1 therefore reducing the probability of long-term survival in the environment.

Although reversion has been observed for viral vaccines, in the case of BPZE1 reversion is highly improbable as DNT was removed by deleting the dermonecrotic toxin gene, and TCT was reduced to background levels by deleting the *B. pertussis ampG* gene and replacing it with that of *E. coli*. A reversion to DNT- and/or TCT-producing BPZE1 could only occur if a gene transfer from a virulent *B. pertussis* (containing DNT and/or *B. pertussis ampG*) was achieved during a period of coacquisition of the 2 microorganisms, which would require the same microbiome niche within the same host simultaneously and for a long enough period to allow for genetic exchange during the replication phase.

Even if virulent *B. pertussis* would co-exist with BPZE1 in the same niche, DNT and/or *ampG* transfer from virulent *B. pertussis* to BPZE1 would be highly improbable, as the genome of *B. pertussis* lacks the genetic information necessary for horizontal gene transfer (Parkhill et al 2003), and no plasmid has ever been detected in *B. pertussis*.

Dispersal

B. pertussis is spread mainly by aerosol formed by coughing or by direct contact with respiratory secretions of infected persons. The GMO has reduced TCT to background levels. Since TCT is potentially responsible for the early pertussis cough syndrome, the GMO is expected to disseminate much less efficiently than the wild strain.

55. The known or predicted environmental conditions which may affect survival, multiplication and dissemination, including wind, water, soil, temperature and pH

A neutral pH and temperature of 37ºC are the ideal conditions for survival in the human respiratory tract.

56. The sensitivity to specific agents

BPZE1 is highly sensitive to antibiotics such as azithromycin or another macrolide used to treat wild-type *B. pertussis* in the clinical setting. Alternative treatment with trimethoprim sulfamethoxazole can be given in the case of macrolide allergy. It is also highly sensitive to usual antiseptics.

Interactions with the environment

57. The predicted habitat of the organism

The human respiratory system is the natural habitat of *B. pertussis*.

58. The studies of the behaviour and characteristics of the organisms and their ecological impact carried out in simulated natural environments, such as microcosms, growth rooms and greenhouses

N/A

59. The capability of post-release transfer of genetic material

a. from the genetically modified organisms into organisms in affected ecosystems

As the genome of *B. pertussis* (and therefore BPZE1) contains no genetic information of horizontal gene transfer systems and no plasmid, transfer of genetic material to other organisms is excluded.

b. from indigenous organisms to the genetically modified organisms

The GMO is not invasive and has no selective advantage in the environment. The potential for exchange of genetic material is virtually non-existent since *B. pertussis* does not harbour plasmids or conjugative transposons. In addition, *B. pertussis* Tohama I strain (background used for the BPZE1 GMO) does not harbour intact prophage genomes and is therefore incapable of producing functional phage particles.

60. The likelihood of post-release selection leading to the expression of unexpected or undesirable traits in the genetically modified organisms

Survival of BPZE1 in the environment is very limited, and it has no selective advantage over the parental strain. The acquisition of unexpected undesirable traits is highly unlikely. To date, 356 healthy volunteers (18 through 50 years old) have been enrolled and exposed to BPZE1 in completed studies. BPZE1 has been generally well tolerated, and there have been no vaccine-related SAEs attributed to BPZE1.

61. The measures employed to ensure and to verify genetic stability, the description of genetic traits which may prevent or minimise dispersal of genetic material and methods to verify genetic stability

The genetic stability of the BPZE1 strain was assessed after 20 and 27 weeks of continuous passaging *in vitro* and *in vivo*, respectively. No genetic or protective difference was observed between passaged bacteria and non-passaged BPZE1, indicating that the attenuated strain is stable.

As the genome of *B. pertussis* (and therefore BPZE1) contains no genetic information of horizontal gene transfer systems and no plasmid, therefore chances of transfer of genetic material to other organisms is remote. (See Feunou-Feunou, P et al. 2008. Vaccine 26:5722-5727).

ILiAD has verified the genetic stability (Study report # IB-100R) by PCR test. The qPCR demonstrated that BPZE1 lyophilised drug product and BPZE1 liquid formulation contain the same genetic inactive pertussis toxin, and no revertant to wildtype pertussis gene at the 2 mutated loci were detected (limit of deletion: 1 active *pt* gene in the presence of 10^6 inactive pertussis toxin genes).

62. The routes of biological dispersal, known or potential modes of interaction with the disseminating agent, including inhalation, ingestion, surface contact and burrowing

The GMO is a non-invasive respiratory bacterium that colonises the upper respiratory tract. Wild-type *B. pertusiss* utilizes humans as the only known reservoir and is spread by close human contact (including aerosol). The strain administered in this trial (BPZE1) is genetically modified to attenuate virulence.

In laboratory settings *B. pertussis* can exist on inanimate surfaces for short periods (3 to 5 days), much shorter than most nosocomial pathogens found in laboratory settings.

It can be eradicated with standard cleaning practices (for example, 70% ethanol, diluted bleach). There is no documentation of *B. pertussis* on animate objects in community settings and it does not exist on plants, in water, or in soil.

63. The description of ecosystems to which the organisms could be disseminated

The only natural habitat of *B. pertussis* is humans. At artificially high doses, the bacteria can be forced to colonise mice, rabbits, primates, and other animals.

64. The potential for excessive population increase of the organisms in the environment

The environment of BPZE1 is the human nasopharynx, where no excessive population increase has been detected in human volunteers receiving either one or two doses of the vaccine: most volunteers having been infected with 10^9 CFU BPZE1. Prolonged colonisation is not observed, and clearance occurs over the subsequent 28 days as is seen with wild-type infections.

Excessive population increase is therefore highly unlikely. Mucosal and systemic immune responses were observed in humans following a single vaccination with BPZE1, with demonstrated durability of response over 9 months. BPZE1 was able to protect against re-colonisation following attenuated challenge with BPZE1 at 3 months after the initial vaccination and this was not observed with aceluar pertussis vaccination (for example, Boostrix).

65. The competitive advantage of the organisms in relation to the unmodified recipient or parental organism or organisms

BPZE1 has no selective advantage over BPSM or Tohama I. BPZE1 colonises the mouse respiratory tract slightly less well than the parental strain.

66. The identification and description of the target organisms if applicable

Identification of the GMO is performed by culture and PCR. The presence of GMO is only applicable to a human's nasopharynx, or in growth media or culture plates.

67. The anticipated mechanism and result of interaction between the released organisms and the target organisms if applicable

There is no planned interaction between the GMO strains and the wild type *B. pertussis.*

68. The identification and description of non-target organisms which may be adversely affected by the release of the genetically modified organisms, and the anticipated mechanisms of any identified adverse reaction

As wild-type *B. pertussis* is a strictly human pathogen, no non-target organism may be adversely affected by the BPZE1 release.

69. The likelihood of post-release shifts in biological interactions or in the host range

The likelihood of post-release shifts in biological interactions is negligible, the likely interactions with nasopharyngeal fauna is as previously discussed.

70. The known or predicted interactions with non-target organisms in the environment, including competitors, prey, hosts, symbionts, predators, parasites and pathogens

N/A

71. The known or predicted involvement in biogeochemical processes

N/A

72. Any other potentially significant interactions with the environment

N/A

Part V: Information on monitoring, control, waste treatment and emergency response plans

Monitoring techniques

73. Methods for tracing the organisms and for monitoring their effects

Air sampling during wild type human challenge did not reveal aerosolised pathogenic organism in the challenge unit (de Graaf et al., CID 2020). Patients with acute respiratory symptoms of *B. pertussis* can be sampled using mid-turbinate or nasopharyngeal swabs or by nasal washings or aspiration and then samples sent for PCR or culture, respectively.

Culture is via standard microbiologic methods (charcoal agar or Bordet Gengou) but *B. pertussis* is fastidious and laboratories needs expertise to achieve success. PCR has become more widespread due to its increased sensitivity and ease of instrumentation methodology.

Culture requires incubation at 37ºC for at least 3 days to elicit colony growth. Silver colored uniform colonies of *B. pertussis* are surrounded by a hemolytic halo ring and can be identified by visual inspection.

In addition to culture, colonies can be analysed using MALDI-TOF as a secondary method of confirmation. Assessing BPZE1 occurs through the same procedures. For PCR amplification the mutational areas are targeted: *ampG* gene, the *dnt* gene or the PTX mutation area.

74. Specificity (to identify the organisms and to distinguish them from the donor, recipient or, where appropriate, the parental organisms), sensitivity and reliability of the monitoring techniques

The GMO is identified by genetic characterisation using PCR and sequencing techniques. PCR analysis of the *ampG* and dnt loci of BPZE1:

Genomic DNA is extracted from isolated colonies and used as template for the PCR using appropriate sense and anti-sense oligonucleotides as described in Feunou et al. Vaccine 2008; 26:5722-5727. The amplified products were analysed by electrophoresis within a 1% agarose gel in TAE buffer containing ethidium bromide and visualised under UV light.

Sequence analysis of the *ptx* locus of BPZE1: The DNA fragments containing the region encompassing the R9K and the E129G mutations of the *ptx*S1 gene are amplified by quantitative PCR from bacterial genomic DNA, using appropriate primers as described by Thalen et al. Vaccines 2020;8:523.

75. Techniques for detecting transfer of the donated genetic material to other organisms

The combination of the microbiological (culture) and molecular methods (PCR) can be used for detecting transfer of donated genetic material to other organisms.

The genome of *B. pertussis* (and therefore BPZE1 as well) contains no genetic information of horizontal gene transfer systems and no plasmids. Therefore, horizontal gene transfer to other organisms is virtually impossible, neither in natural conditions, nor in laboratory conditions.

The resistance to streptomycin and nalidixic acid is due to mutations in the bacterial chromosome, which cannot be transferred to other organisms.

76. Duration and frequency of the monitoring

Subjects will be monitored throughout the study for adverse events at each scheduled visit during the Vaccination and Challenge Phases and during the Safety Follow-up. Reactogenicity will be monitored immediately following vaccination with subjects remaining in the clinic for 30 minutes.

Subjects will then record maximum daily reactogenicity (systemic and nasal or respiratory) in a subject diary for the 7 days following vaccination. If grade 3 toxicity occurs on any day, subjects are to undergo further evaluation.

77. Methods and procedures to avoid and/or minimise the spread of the organisms beyond the site of release or the designated area for use

The vaccine delivery device (for example, MAD) has a conical shape that securely fits into the nostril and delivers an atomised (fine mist) spray within the volunteer's nasopharynx. The vaccination is conducted in the clinic setting under clinical professional guidance.

Subjects remain in the examination room for 30 minutes before being allowed to leave. Immediate reactogenicity is assessed prior to discharge (systemic, nasal or respiratory).

A pilot study has been conducted in neonate baboons (n=3) and their mothers and to date GMO has not been detected in neonate or maternal close physical contact.

78. Methods and procedures to protect the site from intrusion by unauthorised individuals

All investigational products or devices must be stored in a secure area with access limited to the authorised site staff and under physical conditions that are consistent with investigational product-specific requirements. This includes measures to restrict access to unblinded staff that perform drug accountability processes. Study vaccines must be stored in a secure area, at appropriate temperature (for example, refrigerator or freezer), and protected from light and moisture.

The study vaccines may not be used for any purposes other than those outlined in the protocol.

Under no circumstances should the investigator, unblinded drug accountability staff or other site personnel supply study Investigational Product to other investigators or clinics or allow the supplies to be used other than as directed by the protocol without prior authorisation from the sponsor.

The unblinded drug accountability staff must maintain adequate records documenting the receipt, use, loss, or other disposition of the investigational products. All unused medications will be destroyed or returned to the sponsor according to Good Pharmacy and Clinical Practice and the study-specific manuals.

79. Methods and procedures to prevent other organisms from entering the site

The dilutions and preparation of the admixed solutions for administration will be carried out by appropriately qualified staff at the site in accordance with the randomisation schedule, protocol, and pharmacy manual. There will be a 2-person verification process for product assignment or reconstitution and drug accountability log completion to ensure appropriate administration as per randomisation assignment.

Waste treatment

80. Type of waste generated

Clinical and laboratory GMO waste may include nasal sampling supplies, pipette ends, disposable gloves, microbiological waste, vials, syringes, small plastic delivery containers, MAD, and 18G needles. GMO procedures for disposal will be followed. Other procedures for biological wastes will also be managed according to site SOPs and ICH guidelines.

81. Expected amount of waste

The amount of waste will depend on the clinical procedure performed on the day. Each vaccination requires disposal of Glass vial, a syringe, a small plastic luer-lock hub, MAD, and a needle along with the PPE waste generated during the clinical procedure by the site staff.

82. Description of treatment envisaged

Standard Operating Procedures for clinical waste disposal and handling will be followed. (Biohazard waste disposal, followed by incineration or as appropriate according to the local laws)

Emergency response plans

83. Methods and procedures for controlling the organisms in case of unexpected spread

Efficient antibiotics (azithromycin, erythromycin or other macrolide) treatment can be administered. Alternative treatment with trimethoprim sulfamethoxazole can be given in the case of macrolide allergy.

84. Methods, such as eradication of the organisms, for decontamination of the areas affected

Counter tops can be disinfected with common bactericidal cleaners. Pharmacy manual outlines the use of 70% alcohol solution to wipe down reconstitution area.

85. Methods for disposal or sanitation of plants, animals, soils and any other thing exposed during or after the spread

Biohazard containers

86. Methods for the isolation of the areas affected by the spread

Vaccination takes place in clinical exam room setting.

87. Plans for protecting human health and the environment in case of the occurrence of an undesirable effect

Volunteers are closely monitored by medical staff. The whole study conduct will follow instructions and schedule of assessments described in the study protocol, subject to approval from the MHRA and an ethics committee (REC) prior to enrolment of the first volunteer.

Part VI: A description of the methods used or a reference to standardised or internationally recognised methods used to compile the information required by this schedule, and the name of the body or bodies responsible for carrying out the studies

Information on the methods used for construction and detection of the GMO is under items 8 and 16 of this application The final product for the purpose of this clinical trial is manufactured at approved GMP facilities according to the quality dossier, subject to approval from the MHRA.

Only authorised site staff, who have been properly trained for all vaccination procedures, will administer investigational product. The laboratory team are trained in laboratory safety.

References

- Alonso et al. Infect. Immun. 2001; 69:6038-6043
- Antoine et al. J. Mol. Biol. 2005; 351:799-809
- Antoine and Locht. Infect. Immun. 1990; 58:1518-1526
- Cauchi and Locht. Front. Immunol. 2018; 9:2872
- Debrie et al. J. Immunol. 2019; 203:3293-3300
- Feunou et al. Vaccine 2008; 26:5722-5727
- Jahnmatz et al. Lancet Infect Dis 2020; 20:1290-1301
- Kavanagh et al. Clin. Exp. Allergy 2010; 40:933-941
- Li et al. Allergy 2012; 67:1250-1258
- Li et al. J. Virol. 2020; 84:7105-7113
- Lin et al. J. Clin. Invest. 2020; 130 :2332-2346
- Locht et al. J. Infect. Dis. 2017; 216:117-124
- Mielcarek et al. PLoS Pathog 2006; 2:e65
- Parkhill et al. Nat. Genet 2003; 35:32-40
- Simon et al. Bio/Techniques 1983; 1:784-791
- Skerry et al. Clin. Vaccine Immunol. 2009; 16:1344-1351
- Stibitz. Methods Enzymol. 1994; 235:458-465
- Thalen et al. Vaccines 2020; 8:523
- Thorstensson et al. PLoS One 2014; 9: e83449
- Warfel et al. Proc. Natl. Acad. Sci. USA 2014; 111:787-792

Application for consent to release a GMO

Part A2: data or results from any previous releases of the GMO

Give information on data or results from any previous releases of this GMO by you either inside or outside the European Community (especially the results of monitoring and the effectiveness of any risk management procedures)

The BPZE1 GMO has been manufactured according to GMP specifically as a biological medicinal product (attenuated bacteria) for investigational use in humans. The GMO has been released previously in multiple clinical and non-clinical studies (please refer to the section 32)

Part A3: Details of previous applications for release

Give details of any previous applications to release the GMO made to the Secretary of State under the 2002 Regulations or to another Member State under the Deliberate Release Directive 2001/18/EC

Applications with multiple EU countries have been approved for classifying BPZE1 as a Biosafety Level 1 organism, including France, Germany, Belgium, Spain, and Sweden for the purpose of manufacturing and clinical studies.

Part A4: Risk assessment and a statement on risk evaluation

Risk Assessment: environmental impact of the release of the GMOs

To avoid accidental exposure actions are taken to minimise generation of aerosols, since the bacterium is strictly a respiratory tract organism. Persons handling the BPZE1 bacteria should wear gloves and PPE and must wash their hands with a suitable disinfecting soap before touching their skin and eyes.

Effective antibiotic treatment with azithromycin (or an appropriate antibiotic if the subject is allergic to azithromycin) should be given in case of accidental transmission to other humans.

Due to the robust preclinical safety data, BPZE1 has been classified as a Biosafety Level 1 organism by French authorities Republique Francaise Ministere De L'enseignement Superieur Et De La Recherche (French Ministry of Higher Education and Research). Germany, Belgium, Spain, and Sweden have accepted the French Authority's Biosafety Level 1 rating for the purpose of manufacturing and clinical studies.

Risk assessment: factors affecting dissemination

Wild-type *B. pertussis* is spread mainly by aerosol formed by coughing of infected persons. The coughing is potentially induced by the TCT, which is more than 99% reduced in BPZE1. The BPZE1 strain is not expected to induce coughing, therefore transmission is highly unlikely.

Furthermore, neither baboons nor human volunteers infected with doses of BPZE1 up to 10^9 CFU experienced any significant or prolonged BPZE1-related cough, as described in Locht et al. J. Infect. Dis. 2017; 216:117-124, Thorstensson et al. PLoS One 2014;9: e83449, Jahnmatz et al. Lancet Infect Dis 2020;20:1290-1301and Keech, et al. World Vaccine Congress 2020).

The Clinical Study Report of the phase 2b study (n=300 adult subjects) has been submitted to the FDA and is under review. Data from the CSR is available in the BPZE1 Investigator's Brochure for reference. *B. pertussis* has fastidious growth requirements and has limited survival time outside the human body.

Chronic carriage of wild-type *B. pertussis* has not been reported and is therefore not expected and BPZE1 has not been found to be chronically carried in any study to date. No cross-contamination between the subjects was noted when assessing the immunologic outcomes in the previous Phase 1 and 2 clinical trials of BPZE1, nor was any risk to the family members of study subjects reported.

In case of transmission to other humans, accidentally exposed, an efficient treatment against *B. pertussis* is commercially available and is based on administering erythromycin or other macrolides, including azithromycin. BPZE1 has been shown to be sensitive to erythromycin and other macrolides.

The subjects will stay at the study center for at least 30 minutes after administration to observe for any immediate reactogenicity or safety concerns.

In addition, subjects with frequent contact with children less than 1 year of age (such as, parent, childcare worker, nurse) or subjects who live in the same household as individuals with known immunodeficiency or individuals on immunosuppressant therapy, or pregnant women will be excluded from participation in the study as a safety precaution due to the current development stage of the product.

Risk assessment: human health impact

The risks of BPZE1 administration are expected to be minimal and clinically manageable. *B. pertussis* colonisation is strictly limited to respiratory epithelium without dissemination of the bacteria outside the respiratory tract, which also excludes systemic bacteremia of the BPZE1 strain, even in immune-compromised subjects.

B. pertussis has not been shown to be allergenic in any preclinical or clinical studies to date, nor to have any of the excipients in the lyophilised formulation. BPZE1 has been shown to protect against airway inflammation induced by allergens or viral infections in a murine model (Li et al Allergy 2012; 67:1250-1258, Li et al. J. Virol. 2020; 84:7105-7113).

BPZE1 has also been shown to protect against wild type *B. pertussis* infection 3 hours after immunisation in a murine model (Mielcarek et al PLoS Pathog 2006;2: e65) and in baboons (Locht et al. J. Infect. Dis. 2017; 216:117-124). However, there remains a theoretical risk of allergic reaction, as is present with any vaccine product.

The attenuated BPZE1 bacteria colonises the upper respiratory tract slightly less well than wild-type *B. pertussis*. In the most recent (and largest) study to date (Phase 2b) BPZE1 was noted to be cleared from all individuals on Day 78 after vaccination using microbiological culture.

Furthermore, attenuated challenge with BPZE1 at Day 85 demonstrated protection against re-colonisation if BPZE1 was utilized as the vaccine on Day 1 but not if Boostrix was utilised.

In the current school age study, colonisation of BPZE1 is assessed using PCR from samples obtained from the mid-turbinateor nasopharyngeal on Days 7 (safety lead in) and documented clearance on Days 28 and 85 (approximately half of the subjects at each time point).

In the sub-study (n-around 120 subjects) revaccination or attenuated challenge of BPZE1 on Day 85 (open label) with subsequent sampling on days 92 and 99 is designed to demonstrate that vaccination with BPZE1 (but not Boostrix) on Day 1 can avert subsequent colonisation using an attenuated challenge model approach 3 months later. This sub-study design is similar to the design in the adult Phase 2b study.

In the proposed adult Phase 2b study (IB-202P) colonisation of BPZE1 will be assessed using culture from nasal samples and documented clearance on Day 52 (related to Vaccination).

Subsequent sampling in the challenge phase on Days 9, 11 and 14 (relative to Challenge) and Days 16 and 29 (relative to Challenge) are designed to demonstrate that vaccination with BPZE1 on Day 1 (relative to Vaccination) can avert subsequent colonisation of *B. pertussis* during a wild-type virulent challenge model approach 2 to 3 months later.

In summary, the risk assessment for this study shows a very low potential risk for the study subjects and impact associated with administering BPZE1.

Risk assessment: environmental impact

The preliminary risk assessment for this study suggests there is an extremely low risk for potential environmental impact associated with administering the BPZE1 to study subjects.

Risk assessment: monitoring the GMO

Nasal sampling followed by culture detection of *B. pertussis* will be conducted to ensure that the GMO has a limited survival and clears as expected. In all study subjects to date, the GMO has cleared with 45 days, with most subjects having evidence of no colonisation 28 days post-vaccination.

In summary, the GMO is readily sampled and identified, and the colonisation and clearance behavior has been consistent and controlled over a typically less than 28-day duration.

Risk assessment: emergency response

Efficient antibiotics (erythromycin or other macrolide, including azithromycin) treatment can be administered. The GMO has no resistance to macrolides. BPZE1 is resistant to streptomycin and nalidixic acid, which are not used to treat *B. pertussis* infections.

Furthermore, due to the lack of horizontal gene transfer systems in BPZE1, resistance to streptomycin and nalidixic acid cannot be transferred to other organisms.

As such, the risk assessment shows a clear and effective emergency response in the unlikely situation that the bacteria are disseminated to a non-study participant, or the bacteria has prolonged colonisation.

Part A5: Assessment of commercial or confidentiality of information contained in this application. Identify clearly any information that is considered to be commercially confidential. A clear justification for keeping information confidential must be given

N/A

Part A6: Statement on whether detailed information on the description of the GMO and the purpose of release has been published

Make a clear statement on whether a detailed description of the GMO and the purpose of the release have been published, and the bibliographic reference for any information so published.

This is intended to assist with the protection of the applicant's intellectual property rights, which may be affected by the prior publication of certain detailed information, for example, by its inclusion on the public register.

Peer-reviewed publications and issued patents have described the GMO's structure, safety, and genetic stability and use as a vaccine against pertussis

- Alonso et al. Infect. Immun. 2001; 69:6038-6043
- Antoine et al. J. Mol. Biol. 2005; 351:799-809
- Antoine and Locht. Infect. Immun. 1990; 58:1518-1526
- Cauchi and Locht. Front. Immunol. 2018; 9:2872
- Debrie et al. J. Immunol. 2019; 203:3293-3300
- Feunou et al. Vaccine 2008; 26:5722-5727
- Jahnmatz et al. Lancet Infect Dis 2020; 20:1290-1301
- Kavanagh et al. Clin. Exp. Allergy 2010; 40:933-941
- Li et al. Allergy 2012; 67:1250-1258
- Li et al. J. Virol. 2020;8 4:7105-7113
- Lin et al. J. Clin. Invest. 2020;1 30 :2332-2346
- Locht et al. J. Infect. Dis. 2017; 216:117-124
- Mielcarek et al. PLoS Pathog 2006; 2: e65
- Parkhill et al. Nat. Genet 2003; 35:32-40
- Simon et al. Bio/Techniques 1983; 1:784-791
- Skerry et al. Clin. Vaccine Immunol. 2009; 16:1344-1351
- Stibitz. Methods Enzymol. 1994; 235:458-465
- Thalen et al. Vaccines 2020; 8:523
- Thorstensson et al. PLoS One 2014; 9: e83449
- Warfel et al. Proc. Natl. Acad. Sci. USA 2014; 111:787-792